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Retinoic acid controls expression of epidermal transglutaminase at the pre-translational level

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Human epidermal keratinocytes were cultured until sub-confluence in low Ca^{2+} (0.15 mM) serum-free synthetic MCDB 153 medium. Raising the Ca^{2+} concentration to 1.15 mM caused an increase in envelope competence as well as plasma membrane associated transglutaminase (TGm) activity. This increase was not observed when the high Ca^{2+} medium contained retinoic acid. Immunofluorescence studies as well as immunoblotting with the TGm-specific monoclonal antibody B.C1 revealed that retinoic acid inhibits expression of TGm. Isolation and in vitro translation of mRNA with subsequent immunoprecipitation showed that retinoic acid inhibits TGm expression at the pretranslational level.

Keratinocyte; Retinoic acid; Transglutaminase

1. INTRODUCTION

Cultured normal human keratinocytes are able to express two types of transglutaminase, the cytosoluble, ubiquitous tissue type (TGc) and a plasma membrane associated enzyme (TGm) found in the epidermis and other stratifying epithelia [1,2]. TGm is responsible for the synthesis of the cornified envelope in terminally differentiating keratinocytes [1,3–6]. Appearance of TGm, and thus envelope formation is suppressed in the presence of retinoic acid [3,4,7,8] whereas under the same conditions TGc is either not affected (in normal human keratinocytes [7]) or even strongly stimulated (in many other cell types [9,10]).

Even though TGc and TGm have different immunoreactivities and biochemical properties [2], both enzymes catalyse protein cross-linking upon Ca^{2+} activation giving rise to distinct envelope like structures in cultured keratinocytes [11]. It has been speculated very often, but never proven unequivocally that retinoic acid acts by inhibiting the de novo synthesis of TGm rather than by preventing a post-translational modification - one which may, for example, be necessary for the anchorage of the enzyme in the plasma membrane [12].

In the present paper we show by in vitro translation of human keratinocyte mRNA and subsequent immunoprecipitation with the TGm-specific monoclonal antibody B.C1 that retinoic acid controls the expression of this enzyme at the pre-translational level.

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Abbreviations: DMEM, Dulbecco's modification of Eagle's medium containing 5.5 mM glucose and 4 mM glutamine; DTE, Dithioerythritol; EDTA, Ethylenediamine tetraacetic acid; EGF, Epidermal growth factor; FCS, Fetal calf serum; F12, Ham's medium F12; NHK, Normal human keratinocytes; PBS, Phosphate-buffered saline; SDS, Sodium dodecylsulfate; TGc, Cytosoluble transglutaminase; TGm, Plasma membrane associated transglutaminase; Tris, Tris(hydroxymethyl)aminomethane

2. MATERIALS AND METHODS

2.1. *Chemicals*

The MCDB 153 culture medium and bovine pituatary extract were obtained from Clonetics Corporation, San Diego. The other media and fetal calf serum were from Flow Laboratories and GIBCO. [^{35}S]methionine and EN3HANCE were from New England Nuclear. Poly(U) Sepharose was purchased from Pharmacia LKB Biotechnology Inc. and the reticulocyte lysate was supplied by Promega Biotec. The TGm-specific monoclonal antibody B.C1 was raised against the particulate transglutaminase and a generous gift of Dr. S. Thacher, A&M College, Texas.

2.2. *Cells and culture conditions*

Keratinocytes from normal human foreskin were cultured until sub-confluence in low calcium MCDB 153 basal medium (0.15 mM Ca^{2+}), containing 5 mg insulin and 10 μg EGF per liter. This completely defined medium was supplemented with 0.4% bovine pituitary extract. Upon reaching sub-confluence, the cultures were exposed for 6 days to the above medium containing high Ca^{2+} (1.15 mM) in the presence and absence of 3 μM retinoic acid. The medium was changed every second day.

2.3. *Isolation of poly(A$^+$) RNA, in vitro translation and immunoprecipitation*

Total RNA was isolated using the guanidine monothiocyanate extraction procedure of Cathala et al. [13]. Poly(A$^+$) RNA was prepared with poly(U) Sepharose according to [14]. Aliquots (1 μg) of the mRNA were translated in reticulocyte lysates using [^{35}S]methionine as a tracer. The translation products were immunoprecipitated with the TGm-specific B.C1 monoclonal antibody. The culture

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Table 1

Transglutaminase activity and A 23187 ionophore induced envelope formation[a]

Conditions	TGm activity[b]	Envelope synthesis
0.15 mM Ca^{2+}, no retinoic acid	4.3	10%
0.15 mM Ca^{2+} + retinoic acid	0.9	0%
1.15 mM Ca^{2+}, no retinoic acid	47	75%
1.15 mM Ca^{2+} + retinoic acid	5.2	10%

[a]TGm activity and envelope competence were determined as described in [3]

[b]Specific activity is expressed in nmol putrescine bound to dimethylcasein per mg protein per h, at 37°C

medium of the myeloma cell line SP_20 was used as a control. The immunoprecipitates were analysed by SDS gel electrophoresis.

2.4. *Electrophoresis and immunoblotting*

Epidermal TGm was extracted from keratinocytes by homogenizing the cells in a 20 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 1% Nonidet-P40 and 10 μg/ml aprotinin, 1 μg/ml chymostatin, 0.1 mM phenyl methyl sulfonyl fluoride as protease inhibitors. SDS polyacrylamide gel electrophoresis was carried out under reducing conditions as described [15]. A 10% acrylamide gel was used and 75 μg protein were routinely applied to each slot.

For immunoblotting experiments, the proteins were transferred to nitrocellulose paper as described [16]. The sheets were saturated for 1.5 h at room temperature in 10 mM Tris-HCl, pH 7.4, containing 140 mM NaCl and 3% (w/v) bovine serum albumin. They were incubated with the monoclonal antibody B.C1 or with SP20 control supernatant. The sheets were washed and incubated with ^{125}I-labeled anti-mouse Ig $F(ab')_2$ fragment (0.25 mCi/ml). After washing they were exposed for autoradiography to a Kodak XAR-5 film with a DuPont Cronex intensifying screen.

Analysis of the immunoprecipitates was performed on 10% SDS polyacrylamide gels. Fluorography was performed by treating the gels with EN^{3}HANCE. The dried gels were exposed to Kodak XAR-5 films.

2.5. *Indirect immunofluorescence staining*

Immunofluorescence staining was performed as recently described [2]. Cells were fixed and permeabilized with methanol at −20°C for 10 min. After washing with PBS, they were incubated either with B.C1 monoclonal antibody supernatant or with SP_20 supernatant and with fluorescein-conjugated goat anti-mouse IgG (diluted 1:100 in PBS).

3. RESULTS AND DISCUSSION

The shift of sub-confluent normal human epidermal keratinocytes from 0.15 mM Ca^{2+} to medium containing 1.15 mM Ca^{2+}, caused, within 6 days, a remarkable increase in TGm activity. Furthermore, 75% of the cells developed envelope competence, i.e. they synthesized cornified envelopes after ionophore induction.

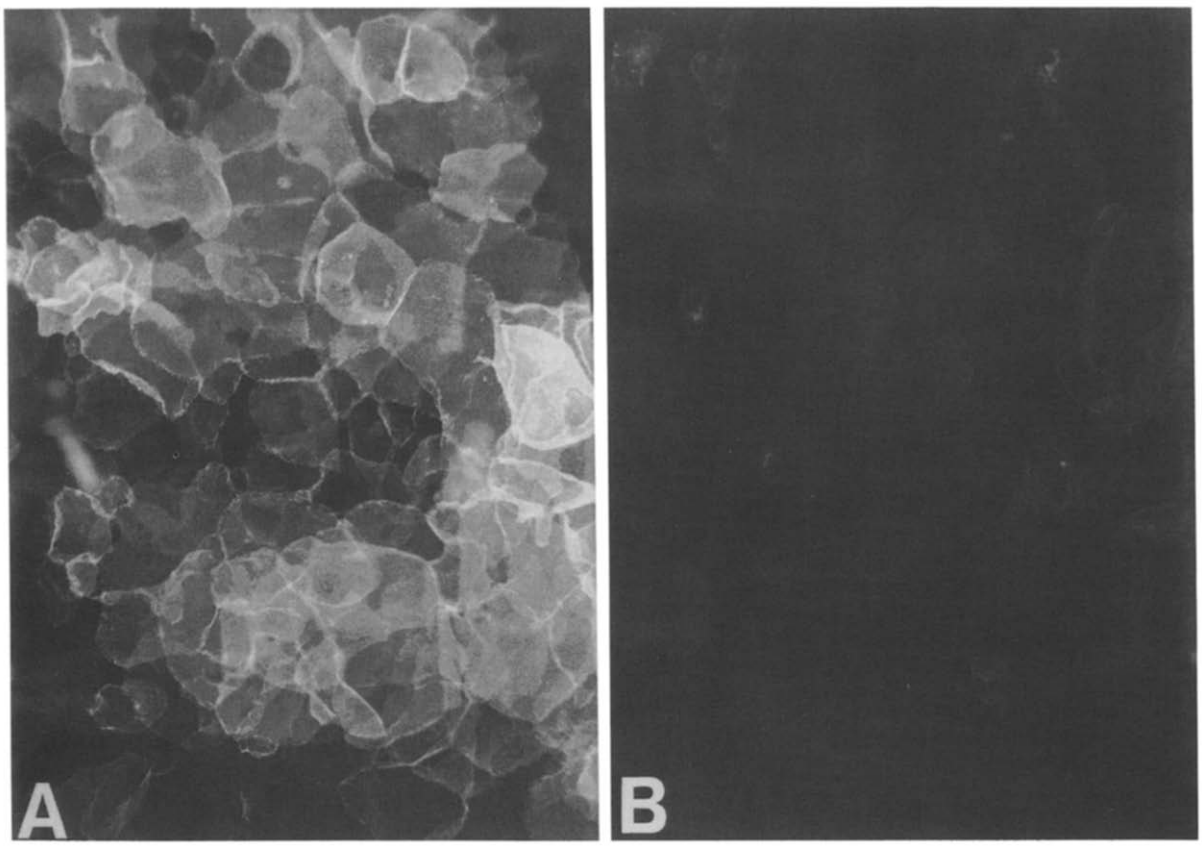

Fig.1. Immunofluorescence staining with the TGm-specific monoclonal antibody B.C1 of control (A) and retinoic acid-treated (B) human keratinocytes 6 days after the shift to high Ca^{2+} (1.15 mM).

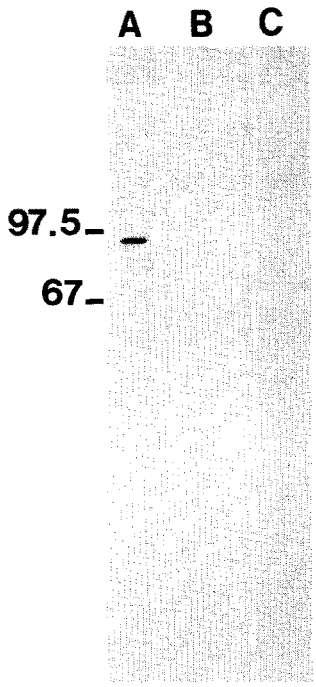

Fig.2. Immunoblot with the TGm-specific monclonal antibody B.C1 after electrophoretic separation of detergent soluble proteins (75 μg/lane) of control (lane A) and retinoic acid treated (lane B) human keratinocytes. Lane C shows the result obtained with the SP_20 control supernatant.

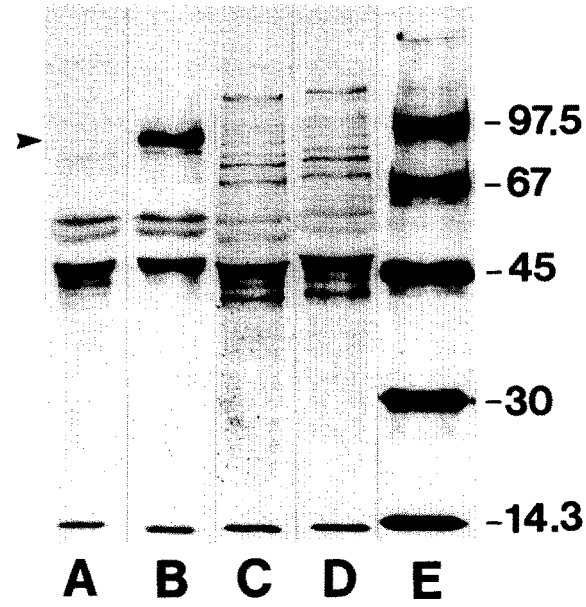

Fig.3. Electrophoretic analysis of proteins obtained after immunoprecipitation with either SP_20 supernatant (lanes A and C) or the TGm-specific monoclonal antibody B.C1 (lanes B and D) of the in vitro translation products of mRNA isolated from control (lanes A and B) or retinoic acid treated (lanes C and D) human keratinocytes. The arrow indicates position of TGm and lane E presents the molecular weight markers.

However, when the high Ca^{2+} medium contained 3 μM retinoic acid, TGm activity did not increase and only 10% of the cells were envelope competent. These results are summarized in table 1.

Immunofluorescence studies using the TGm-specific monoclonal antibody B.C1, revealed the virtual absence of any labeling in retinoic acid treated cells, whereas the plasma membranes of control cells were intensively stained (fig.1A to be compared with fig.1B).

To exclude the possibility that in retinoic acid treated cells, masking of TGm accounted for this finding, a detergent soluble protein extract was prepared from control and retinoic acid treated cells as described in Materials and Methods. The immunoblots obtained after electrophoretic separation of these proteins revealed the presence of a substantial amount of TGm in control cells, whereas TGm was hardly detectable in extracts obtained from retinoic acid treated cells (fig.2, lane A to be compared with lane B).

The above results obtained by immunofluorescence staining and immunoblotting indicate that retinoic acid does not interfere with the TGm-activity but rather inhibits expression of this enzyme.

To find out at which level retinoic acid affects TGm expression, mRNA from control and retinoic acid-treated cells was purified and translated in vitro in reticulocyte lysates. The translation products were immunoprecipitated with the TGm-specific B.C1 monoclonal antibody and electrophoretically separated. Fig.3 shows that the control cells but not the retinoic acid-treated cells contained translatable mRNA coding for TGm (lane B to be compared with lane D).

From these results we conclude that retinoic acid interferes at the pre-translational level, very likely by inhibiting transcription of the TGm gene. The effect of retinoic acid is most probably mediated by the recently described nuclear retinoic acid receptors [17–20]. However, other retinoic acid induced mechanisms that involve premature termination of the transcription process or the degradation of mRNA [21,22] cannot be excluded.

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